Discovery of a novel putative atypical porcine pestivirus in pigs in the USA

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Pestiviruses are some of the most significant pathogens affecting ruminants and swine. Here, we assembled a 11 276 bp contig encoding a predicted 3635 aa polyprotein from porcine serum with 68 % pairwise identity to that of a recently partially characterized Rhinolophus affinis pestivirus (RaPV) and approximately 25–28 % pairwise identity to those of other pestiviruses. The virus was provisionally named atypical porcine pestivirus (APPV). Metagenomic sequencing of 182 serum samples identified four additional APPV-positive samples. Positive samples originated from five states and ELISAs using recombinant APPV Erns found cross-reactive antibodies in 94 % of a collection of porcine serum samples, suggesting widespread distribution of APPV in the US swine herd. The molecular and serological results suggest that APPV is a novel, highly divergent porcine pestivirus widely distributed in US pigs.

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The genus Pestivirus includes four recognized species: bovine viral diarrhea virus type 1 (BVDV-1), bovine viral diarrhea virus type 2 (BVDV-2), classical swine fever virus (CSFV) and border disease virus (BDV) (Simmonds et al., 2011). In addition, several unassigned atypical pestiviruses have been characterized (giraffe pestivirus, antelope pestivirus, HoBi virus and Bungowannah virus). CSFV is a significant swine pathogen (Chander et al., 2014). Infection with highly virulent strains leads to high morbidity and mortality while infection of the fetus with low virulent strains results in fetal death or birth of persistently infected animals. While cattle represent the natural host of BVDV, infections in numerous domestic and wild animals have been documented (Becher et al., 2003). BVDV infection in swine is typically subclinical (Tao et al., 2013).

In 2003, a divergent pestivirus, Bungowannah virus (Bungo), was isolated from a farm in Australia exhibiting an outbreak of sudden death in three- to four-week-old pigs and an increase in stillborn fetuses (Kirkland et al., 2007). Pathologically, multifocal non-suppurative myocarditis with myonecrosis was observed. Phylogenetic analysis found Bungowannah virus to be the most divergent pestivirus and antigenically it failed to react with pan-reactive pestivirus monoclonal antibodies.

More recently, two novel pestivirus genomes were identified by next-generation sequencing (NGS). Analysis of the virome from the bat species Rhinolophus affinis in China identified a 5 kb contig with 32 % amino acid sequence identity to known pestiviruses (Wu et al., 2012). Metagenomic sequencing of Norway rats in New York City also identified a highly divergent pestivirus genome (Norway rat pestivirus, NRPV) which shared a maximum of 60 % amino acid identity with known pestivirus polyproteins (Firth et al., 2014). These two newly described pestivirus sequences are the first identified in species outside the order Artiodactyla and suggest a wider pestivirus host range. Nothing is known about the ability for these viruses to cause disease.

Metagenomic sequencing was performed on a sample that was quantitative reverse transcription PCR (qRT-PCR) positive for porcine reproductive and respiratory syndrome virus (PRRSV) as part of a PRRSV metagenomic sequencing project as previously described (Hause et al., 2015; Neill et al., 2014). Following subtraction of reads mapping to the host Sus scrofa, reads were assembled de novo into 2167 contigs and analysed by BLASTN. Six contigs returned
Identiﬁcation of a novel porcine pestivirus

Our analysis of a putative porcine pestivirus (APPV) indicates a novel member of the Pestivirus genus. It was provisionally named atypical porcine pestivirus (APPV). Putative APPV mature protein sequences were identiﬁed by alignment with reference pestivirus genomes and known cleavage sites (Table S1, available in the online Supplementary Material) as determined for CSFV strain Alfort; however, these require conﬁrmation experimentally (Rumenapf et al., 1993; Stark et al., 1993; Vilcek et al., 2005). Virus isolation was attempted on MARC-145, Vero, Vero 76, HCT-8, BT, MDBK, ST, PK15 and MDCK cell lines. No cytopathic effect (CPE) was evident in any cell line. Viral titres were monitored by E2 RT-PCR. Following two passages on cells, all samples were qRT-PCR negative.

The 5′-untranslated region (UTR) determined encompassed 123 bp, which is considerably shorter than those for other pestiviruses (~370–498 bp). Attempts to verify the termini sequences by random ampliﬁcation of cDNA ends (RACE) failed, likely due to insuﬃcient virus titre (Ct ~30). Additional samples qRT-PCR-positive for APPV with lower Ct values were subsequently identiﬁed; however, insuﬃcient sample was available to attempt RACE. It is likely that the 5′-UTR sequence is incomplete given the conservation of the size of the pestivirus 5′UTR; however, this needs to be demonstrated experimentally. The 245 bp of sequence determined for the 3′UTR is consistent with the length of other pestivirus 3′UTR’s (~200–500 bp) but further experimentation is needed to demonstrate its completeness.

The Npro protein is unique to the genus Pestivirus and is a non-structural autoprotease (Stark et al., 1993). Npro catalyses self-cleavage from the polyprotein between Cys168 and Ser169. The conserved Npro catalytic site consisting of Glu22, His49 and Cys69 was identiﬁed in APPV at Glu 20, His69 and Cys89 by pairwise alignment with known pestiviruses (Rumenapf et al., 1998). Despite conservation of the catalytic and cleavage sites, the Npro protein sequence had no signiﬁcant similarity to any known proteins by BLASTP analysis and had only 9–18% pairwise amino acid identity to other Npro sequences (Table S2).

Pestiviruses encode a small, basic core protein (C) which possesses RNA chaperone activity and plays a role in RNA packaging into virions (Ivanyi-Nagy et al., 2008; Murray et al., 2008). At 111 aa in length, the C protein of APPV is slightly larger than other pestivirus C proteins (97–102 aa) but has a predicted isoelectric point of 10.4, similar to other pestiviruses.

Pestiviruses encode three envelope glycoproteins, E1, E2 and Erns. The Erns protein, found only in pestiviruses, is the only known viral structural protein with a RNase T2 domain with uridylate speciﬁcity (Schneider et al., 1993). The RNase T2 domain was identiﬁed in the APPV Erns region of the genome from aa 319 to 373 using the National Center for Biotechnology Information (NCBI) Conserved Domain Database. The Erns protein also acts to inhibit interferon production by degradation of viral dsRNA produced during viral replication (Iqbal et al., 2004). As the T2 RNase superfamily domain was identiﬁed in Erns, it appears likely that APPV degrades viral dsRNA to prevent a cellular interferon response despite its 32.9–39.0% identity to other pestivirus Erns proteins.

### Table 1. BLASTP analysis of the twelve putative mature proteins and the polyprotein of atypical porcine pestivirus (APPV)

<table>
<thead>
<tr>
<th>Region</th>
<th>Size (aa)</th>
<th>Best BLAST hit (accession number)</th>
<th>Query coverage (%)</th>
<th>E value</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Npro</td>
<td>180</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>C</td>
<td>111</td>
<td>BVDV (NP_776260)</td>
<td>89</td>
<td>1e⁻⁵</td>
<td>37</td>
</tr>
<tr>
<td>Erns</td>
<td>210</td>
<td>CSFV (AFKE56244)</td>
<td>97</td>
<td>2e⁻⁴⁷</td>
<td>43</td>
</tr>
<tr>
<td>E1</td>
<td>199</td>
<td>BVDV (ACV83744)</td>
<td>98</td>
<td>1e⁻²⁷</td>
<td>32</td>
</tr>
<tr>
<td>E2</td>
<td>241</td>
<td>RaPV (AKF85014)</td>
<td>100</td>
<td>4e⁻⁸⁴</td>
<td>54</td>
</tr>
<tr>
<td>P7</td>
<td>64</td>
<td>RaPV (AKF85014)</td>
<td>100</td>
<td>2e⁻¹⁹</td>
<td>67</td>
</tr>
<tr>
<td>NS2</td>
<td>314</td>
<td>RaPV (AKF85014)</td>
<td>100</td>
<td>4e⁻¹²⁵</td>
<td>60</td>
</tr>
<tr>
<td>NS3</td>
<td>687</td>
<td>RaPV (AKF85014)</td>
<td>100</td>
<td>0</td>
<td>74</td>
</tr>
<tr>
<td>NS4a</td>
<td>67</td>
<td>RaPV (AKF85014)</td>
<td>100</td>
<td>1e⁻²⁰</td>
<td>61</td>
</tr>
<tr>
<td>NS4b</td>
<td>339</td>
<td>RaPV (AKF85014)</td>
<td>76</td>
<td>4e⁻¹¹⁰</td>
<td>76</td>
</tr>
<tr>
<td>NS5a</td>
<td>472</td>
<td>BDV (AHM88396)</td>
<td>45</td>
<td>2e⁻⁶</td>
<td>26</td>
</tr>
<tr>
<td>NS5b</td>
<td>751</td>
<td>CSFV (AAT85641)</td>
<td>89</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Polyprotein</td>
<td>3635</td>
<td>RaPV (AKF85014)</td>
<td>46</td>
<td>0</td>
<td>68</td>
</tr>
</tbody>
</table>

http://jgv.microbiologyresearch.org
E1 and E2 form heterodimers on the virus surface that are crucial for viral entry into cells (Ronecker et al., 2008). The E2 protein is also immunodominant and possesses neutralization epitopes and consequently is the pestivirus protein that exhibits the greatest amount of diversity (Ridpath and Bolin, 1997; Tijssen et al., 1996). Both proteins displayed less than 31 % identity to other pestiviruses; however, E2 was 54 % identical to the recently described RaPV (Table S2). It should be noted that only 5.1 kb of the RaPV genome is known, encompassing the E2, P7, NS2, NS3 and NS4α regions of the genome (Wu et al., 2012). Interestingly, the E2 proteins for APPV and RaPV were significantly shorter (241 and 244 aa, respectively) than those for all other pestiviruses (373–378 aa). The deletion accounting for the smaller size of the APPV E2 protein was located at the N terminus. The cell tropism of BVDV is determined by the E2 protein, which binds to its receptor CD46 (Liang et al., 2003; Maurer et al., 2004). With the exception of NRPV1 and RaPV, where virus has not been isolated, all pestiviruses are readily cultured in cells in vitro. Further experimentation is needed to determine if the substantial reduction in E2 size and only ~30 % identity to non-RaPV pestivirus E2 proteins has resulted in a change in receptor utilization.

The P7 protein is a small hydrophobic peptide 61–2 aa in length that functions as a viroporin that is essential for virus production in vitro and virulence in vivo (Glade et al., 2012). The P7 of APPV was 67 % identical to the P7 of RaPV and lacked significant similarity to any other proteins in GenBank.

NS2 is a cysteine autoprotease responsible for cleavage of NS2 from NS3 (Lackner et al., 2004). A search of APPV NS2 failed to identify conserved protease domains. BLASTP analysis of the APPV NS2 found significant similarity only to RaPV NS2 (60 % identity). The active site of NS2 of BVDV has a His1447 and Cys1512 and is thought to include a glutamate residue between aa 1447 and 1512 (Lackner et al., 2004). Multi-sequence alignment identified a conserved His1237 and Glu1253 in the APPV NS2; however, the conserved cysteine residue of the catalytic triad could not be identified. One candidate cysteine residue at position 1280 was identified. NS3 is a chymotrypsin-like serine protease catalysing both cis- and trans-cleavage (Lamp et al., 2013; Ryan et al., 1998). The APPV NS3 protein shared relatively high identity to the NS3 of RaPV (74 % identity). Conserved domains identified include a DEAD-like helicase from aa 1547 to 1672 and a pestivirus peptidase S31 domain from aa 1320 to 1530. NS4α, NS4b, NS5α and NS5β are all involved with pestivirus replication (Behrens et al., 1998). NS4α was 61 % identical to RaPV NS4α and had 29–33 % identity to NS4α of other pestiviruses. NS4b and NS5b shared a similar amount of identity to those of other pestiviruses (36–45 %) while NS5a was less conserved (12–17 %).

Phylogenetic analysis was performed in MEGA 6.06 (Tamura et al., 2013). Sequences were aligned by CLUSTAL W, and phylogeny was inferred using the maximum-likelihood method with tree topology verified by 500 bootstrap replicates. Phylogenetic analysis was performed on the polyprotein, Npro, Erns and NS5 proteins (Fig. 1). APPV and RaPV formed a distinct cluster for proteins where RaPV sequence was available. The APPV/RaPV cluster represented a highly divergent lineage of pestiviruses that evolved from an ancestral pestivirus. For Npro and Erns, APPV proteins represented a highly divergent lineage.

As part of a project investigating PRRSV genetic diversity using metagenomic sequencing of swine serum samples, a total of 182 PRRSV qRT-PCR-positive samples were sequenced using viral metagenomic methodology. Templated assembly was performed on these 182 samples using the APPV genome. Reads mapping to the APPV genome were identified in five samples. To confirm these results, two Taqman qRT-PCR assays were designed, targeting either the E2 or Erns region of the genome. The original sample, 146, was positive for both assays with C_{\text{t}} values of 25.1 and 30.5 (1 : 10 dilution of RNA), respectively. Sample 208 was only tested in the E2 qRT-PCR assay due to insufficient sample and was positive with C_{\text{t}}=19.3. Samples 51 and 98 were positive for both assays with C_{\text{t}}=33.2 and 36.2 for the E2 assay and C_{\text{t}}=33.9 and 34.1 for the Erns assay, respectively. Sample 28 was negative in the E2 assay but was positive with a C_{\text{t}}=32.3 for the Erns assay. We also screened a collection of 292 PRRSV qRT-PCR-negative serum samples. Using the E2 qRT-PCR assay all samples were negative.

For samples 208 and 28, there was sufficient read coverage for the E2 region of the genome to assemble complete and partial E2 sequences, respectively. The E2 sequence for sample 208 was identical to that for sample 146. For sample 28, a 197 aa portion of the E2 protein was assembled that was 88 % identical to that of sample 146. The five positive samples were collected in 2014 from Nebraska, Arizona, North Carolina, Minnesota and Kansas, suggesting widespread distribution of APPV in the US swine herd. The finding of only 88 % amino acid identity for the partial E2 sequence of sample 28 also suggests significant genetic diversity is present in APPV and is likely the reason for the failure of the E2 assay to detect sample 28.

Two APPV Erns peptides were expressed as 6 × -histidine fusion proteins in Escherichia coli and purified by affinity chromatography. An ELISA was developed to detect antibodies in swine sera that cross-react with APPV Erns, similar to that previously described for CSFV (Lin et al., 2005). A collection of 78 PRRSV qRT-PCR-positive serum samples collected from multiple states, samples from three different production sites (farms 1, 2 and 3) and 15 specific-pathogen-free (SPF) pigs were assayed on the APPV Erns ELISA. Mean group OD405 nm values ranged from 0.48 to 1.25 (Table 2). All group mean OD values of sample 208 were significantly different from one another with the exception of farm 3 and the negative control SPF pig group by ANOVA and Tukey–Kramer HSD (P<0.05). The value of the mean negative control plus three standard deviations has been used to determine the negative cut-off for ELISAs.
Applying this formula to the negative controls yielded a cut-off value $OD > 0.72$. For the PRRSV qRT-PCR-positive samples, 73/78 (94%) of samples were positive. For samples from a single site, 90/90 (100%) samples from farm 1, 46/48 (96%) samples from farm 2, and 0/30 (0%) of the samples from farm 3 were positive. These serological results require substantiation by additional assays including control samples with known serological status.

The presence of both Npro and Erns proteins and overall modest similarity to known pestiviruses suggest APPV should be assigned taxonomically to the genus *Pestivirus*. Phylogenetic analysis found that APPV is most closely related to RaPV and the divergence between APPV and RaPV was similar or greater than between recognized pestivirus species BDV, BVDV and CSFV. We consequently propose that APPV and RaPV represent novel species in the genus *Pestivirus*. The finding of a divergent pestivirus that is widely distributed in pigs in the US raises numerous questions. Is APPV a newly emerged swine virus or has it circulated unrecognized for some time? Does APPV

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**Fig. 1.** Phylogenetic trees of pestivirus (a) polyprotein, (b) Npro, (c) Erns and (d) NS3. Maximum-likelihood analysis in combination with 500 bootstrap replicates as implemented in MEGA 6.06 (Tamura et al., 2013) was used to derive trees based on the predicted protein sequences. Bars, number of amino acid changes. Bootstrap values are shown at nodes as percentages. Abbreviations are as follows with GenBank accession numbers in parentheses: BDV, border disease virus (NC003679); RPV, reindeer pestivirus (AAF02524); CSFV, classical swine fever virus (NC002657); GPV, giraffe pestivirus (NP620053); BVDV, bovine viral diarrhea virus (NC001461); Hobi, HoBi virus (BAO04453); APV, antelope pestivirus (NC024018); Bungo, Bungo-wannah virus (NC023176); NRPV, Norway rat pestivirus (NC025677); RaPV, Rhinolophus affinis pestivirus 1 (JQ814854); APPV, atypical porcine pestivirus (KR011347).

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**Table 2.** Detection of antibodies cross-reactive to atypical porcine pestivirus Erns peptides produced in *E. coli*

PRRSV qRT-PCR positive samples were collected from multiple sites representing at least seven US states. Sera in other groups were collected from a single site.

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>n*</th>
<th>Mean OD†</th>
<th>Standard deviation (OD)</th>
<th>Positive samples (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRRSV qRT-PCR-positive</td>
<td>78</td>
<td>1.25</td>
<td>0.33</td>
<td>73/78 (94)</td>
</tr>
<tr>
<td>Farm 1</td>
<td>90</td>
<td>1.06</td>
<td>0.15</td>
<td>90/90 (100)</td>
</tr>
<tr>
<td>Farm 2</td>
<td>48</td>
<td>0.96</td>
<td>0.12</td>
<td>46/48 (96)</td>
</tr>
<tr>
<td>Farm 3</td>
<td>30</td>
<td>0.57</td>
<td>0.06</td>
<td>0/30 (0)</td>
</tr>
<tr>
<td>Specific-pathogen-free pigs</td>
<td>15</td>
<td>0.48</td>
<td>0.08</td>
<td>0/15 (0)</td>
</tr>
</tbody>
</table>

*Number of serum samples.
†Groups not connected by the same letter are significantly different ($p < 0.05$).
‡A cut-off of $OD > 0.72$ was used to determine positivity.
infection of swine result in pathology, and what clinical symptoms, if any, result? The evolutionary relationship to RaPV also draws into question our understanding of the ecology, host range and natural reservoirs of pestiviruses, warranting further investigation.

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References


